

Claims 22 and 23, line 1 of each, change "enhancing" to
--inducing--

REMARKS

Reconsideration of this application is requested in view of the amendments to the specification and claims and the remarks presented herein.

The claims in the application are claims 1 to 14 and 17 to 25. Non-elected claims 15, 16 and 26 to 28 have been cancelled but Applicants reserve the right to file a divisional application directed thereto.

Applicants are in the process of requesting new formal drawings to be prepared and the specification has been amended to cancel reference to the references by number and have instead inserted the various references at the appropriate place. Moreover, pages 31 to 34 listing the references has been cancelled.

With respect to the Examiner's remarks that the Abstract of the Disclosure was not provided, Applicants wish to call to the Examiner's attention that the Abstract was provided as the page before page 1. However, Applicants are submitting a copy of the said Abstract herewith. In addition, the grammatical and typographical errors noted by the Examiner have been corrected.

With respect to the use of trademarks in the specification, it is believed that all of the trademarks have been capitalized as required by the Patent Office rules.

Claims 1, 5, 7, 19 and 22 to 23 were rejected under 35 USC 112, second paragraph, as being indefinite. The Examiner objected to the phrase "such as" and the use of the word "type" in claim 7. The term "in particular" was objected to as well.

Applicants respectfully traverse these grounds of rejection since the amended claims are believed to properly define the invention. The Examiner's kind suggestions have been adapted where appropriate and the claims no longer use the word "type" or the phrase "such as" or "in particular". The B-epitope has now been properly set forth in the claims. Therefore, the amended claims are believed to properly define the invention and withdrawal of these grounds of rejection is requested.

Claims 1 to 5, 12, 13, 17, 18 and 22 to 25 were rejected under 35 USC 102 as being anticipated by or under 35 USC 103 as being obvious over the Lett et al reference. Claims 1 to 5, 10, 11, 17 to 19, 22, 24 and 25 were rejected under 35 USC 103 as being obvious over the Lett et al reference taken in view of the Tam patents. Claims 6 to 9, 14, 20 and 21 were rejected under 35 USC 103 as being obvious over the Lett et al reference taken in view of the Tam patents and in further view of the Fung et al reference.

The Examiner states that the Lett et al reference shows on page 2646, column 1, a carbohydrate peptide conjugate comprising a carrier, a dendrimeric polylysine attached thereto 8 individual T epitope peptides and a carbohydrate T-cell independent moiety. The Examiner also states that Lett et al shows that the carbohydrate moiety is a T-cell independent epitope which would teach one skilled in the art that the carbohydrate moiety is a B-cell epitope. The Tam patents are cited to show that conjugates with several identical or different antigenic products of T-cell antigens and B-cell antigens joined to a dendritic polylysine would generate extremely high antibody titers. The Examiner concludes that the invention would be obvious therefrom.

Applicants respectfully traverse these grounds of rejection since it is deemed that the Lett et al reference, whether taken alone or in combination with the secondary and tertiary references, would not suggest Applicants' invention to one skilled in the art. Claim 1 has been modified to clearly state that the carbohydrate moiety of the claimed carbohydrate peptide conjugate as a defined chemical structure that can be precisely described which is equivalent to the term "synthetic". The Mannan-Rich polysaccharide derived from *Saccharomyces cerevisiae* used by Lett et al does not have a defined chemical structure as it clearly results from the method used for its extraction and purification.

For the purification of the Mannan-Rich polysaccharide derived

from *Saccharomyces cerevisiae*, Applicants refer an article by Lett et al, *Infection and Immunity*, Vol. 62 (3) (1994), pg. 782-785, a copy of which is enclosed herewith for the Examiner's convenience. On page 786 of the 1994 article by Lett et al, under the section named Bacterial Strains and Antigen Preparation, there is disclosed in lines 9 to 11 that the commercially available Mannan extract from *Saccharomyces cerevisiae* is treated by pronase before the coupling. It is therefor clear that the Mannan polysaccharide from *S.cerevisiae* used by Lett et al in the 1995 reference is not structurally defined as it is in the case of the polysaccharide of Applicants' claims.

As disclosed in both articles by Lett et al, the Mannan raw material was provided by Sigma Chemicals Company and Applicants are submitting herewith an extract of the Sigma Chemicals Company commercial catalog which contains two references for Mannan derived from *S.cerevisiae* defined as M7504 and M3640. The Mannan material under reference M7504 has been prepared by the method disclosed in an article of Haworth et al from the *Journal Chemical Society*, a copy of which is enclosed herewith for the Examiner's convenience. According to the experimental procedure described in the Haworth et al reference, baker's yeast was boiled in sodium hydroxide solution before being centrifuged to obtain three fractions which are further processed before precipitation of crude Mannan by the addition of alcohol as indicated on page 787. The Lett et al article discloses a multiple antigen peptide (MAP) consisting of a

polylysine core matrix bearing 8 radially branched copies of peptide "VAPNYEKEPT" corresponding to amino acids 861 to 870 and the first proline-rich repeat of protein SR from Streptococcus mutans to which peptide has been conjugated with respectively different polysaccharides. Lett et al has shown that when associated with liposomes which are then administered to rats, the conjugates were able to induce in immunized animals a salivary antipolysaccharide IgA memory. Lett et al has made use of two types of polysaccharide raw materials for conjugation to the MAP construct that they disclose.

The first polysaccharide material used by Lett et al for synthesizing the MAP construct consists of a mixture polysaccharide obtained from Streptococcus mutans cells of the strain OMZ175 (SERO group f) which has been prepared by the method disclosed by Benabdelmoumene et al which is disclosed on page 2646. Applicants are submitting herewith a copy of the said reference for the Examiner's convenience.

This article discloses the method of preparation of polysaccharide extract from Streptococcus mutans OMZ175 (SERO type f) on page 3262, left-hand column, first paragraph.

Briefly, the polysaccharide antigen was prepared from lyophilized Streptococcus mutan cells by (1) harvesting the material present in the supernatant of previously autoclaved

Streptococcus mutan cells, (2) dialysing and lyophilizing said material before a chromatographic separation of different fractions and pulling the fractions containing only carbohydrate. This method of preparation of Streptococcus mutan polysaccharides clearly shows that the resulting polysaccharide material consists in fact of a mixture of polysaccharides of various chemical and structural compositions and in any case, of a polysaccharide of a defined structure.

The second polysaccharide used by Lett et al for conjugation to the MAP construct consists also of a polysaccharide extracted from microorganisms. A chemical analysis of the resulting crude mannan from *S.cerevisisiae* was performed by fractionation after dissolution in a mixture of ether and acetone as well as by hydrolysis of methylated yeast mannan as indicated on page 789.

The crude mannan obtained by the Haworth method can be separated into three different fractions which demonstrate that the raw material is a mixture of three different molecules endowed with distinct physiochemical properties and which chemical structure is not precisely defined. Throughout the Haworth article, the authors affirm that "the size of this molecule (the mannan material) is unknown but it is probably large". According to the Haworth article, the mannan obtained by the method disclosed therein may have several possible structural formulae which may be divided into two main structural formulae indicated as I and II. It appears

that the said formulae are purely hypothetical and based only the results of hydrolysis reactions performed on the raw material. It clearly appears from the Haworth teaching that the yeast mannan used by Lett et al is not a polysaccharide of a defined chemical structure but is rather a mixture of at least three mannan compounds of an undetermined size and of an undetermined chemical structure.

The mannan material from *S.cerevisiae* sold by Sigma Chemicals under M3640 specifically refers to the preparation method disclosed in the 1957 Barker et al reference from the Chemical Industry, a copy of which is enclosed for the Examiner's convenience. The article discloses a method for extracting mannan material from yeast essentially by selective precipitation with the cetavlon detergent. After qualitative cetavlon precipitations of various polysaccharides in borate buffer, the Barker et al article states that 250 ml of yeast mannan could be obtained. It clearly appears that the mannan fraction obtained by the Barker et al process has not undergone any chemical analysis in view of determining its precise chemical structure. Moreover, it was known from the Haworth article that the baker's yeast mannan was not a single material but, rather, a composite mixture of different types of mannan. It results that the yeast mannan fraction of the Barker et al reference was in fact a mixture of various mannan polysaccharides of different sizes and may be of different chemical compositions. It is very clear from the above that the MAP

construct described by Lett et al did not contain a polysaccharide of a defined chemical structure but, rather, a polysaccharide of an undefined size and composition.

In contrast to the MAP construct described by Lett et al, the carbohydrate peptide conjugate of Applicants' invention comprises at least one carbohydrate moiety of a defined chemical structure attached to a carrier of dendrimeric polylysine and this essential feature of Applicants' carbohydrate peptide conjugate is fully supported by the discussion in the application which discloses in detail the synthesis of MAG construct. Example 1 of Applicants' disclosure describes a synthesis of a glycoconjugate and as indicated in lines 3 to 9 of page 13 of the application, the synthesis of the Tn antigen has been performed by chemical reaction starting from tri-O-acetyl-D-galactal and that N-(fluorophenyl methoxy carbonyl)-L-serine tertiary butyl ester has been used for a Koenigs Knorr reaction with 3,4,6-tri-O-acetyl-2-azido-2-deoxy-B-D-galactopyranosyl chloride which has been further processed.

The use of a structurally defined or synthetic carbohydrate moiety on a MAP construct was neither disclosed nor suggested by the Lett et al reference. In contrast, Lett et al believes that the use of polysaccharides of non-bacterial or yeast origin but of undefined chemical structure is completely satisfactory to induce the specific immune response sought. In this regard, a particularly relevant portion of the Discussion heading of Lett et

al shows that the authors wrote "Taken together, these results show that peptide 3 and MAP are immunogenic and act as carriers for T-cell independent antigens. These results were confirmed with another polysaccharide, a mannan from *S.cerevisiae* which has been implicated as the key component in many bacterial, fungal and viral infections. Furthermore, the antibody induced by the four conjugates reacted with either *Streptococcus mutans* or *Saccharomyces cerevisiae* whole cells making such conjugates attractive for developing both antiprotein and antipolysaccharide antibodies at the mucosal level." See the left-hand column, first paragraph of page 2650.

Lett et al merely suggests to carry out further experiments using the same MAP constructs that they disclose but with animals of different major histocompatibility complex haplotypes to confirm the efficiency of glycopeptides with MAP as safe vaccines against several infectious diseases as can be seen from the last full paragraph of page 2650. It clearly flows from the teaching that Lett et al has never envisaged to synthesized MAP constructs containing a carbohydrate moiety of a defined chemical structure.

Moreover, the biological activity of the MAP construct of Lett et al has only been shown when these conjugates were used under a form associated with liposomes which implicitly suggests to one skilled in the art that a good presentation of the MAP constructs of Lett et al to the immune system can be obtained only when said

conjugates are exhibited in multiple copies on the outer layer of the liposome. In contrast, Applicants' invention shows that the new conjugates they have synthesized comprise a structurally defined or synthetic carbohydrate moiety which can induce a specific immune response when they are administered to the body under a free form and not in liposomes in the absence of an adjuvant of immunity. Therefore, Applicants' carbohydrate peptide conjugates are novel and in no way anticipated or rendered obvious by the Lett et al reference.

The Tam patents in no overcome the deficiencies of the Lett et al reference. The Tam patents allegedly disclose a carbohydrate conjugate with several different T-cell epitope peptides or B-cell carbohydrates attached to the core polylysine. It must be pointed out that the '490 Tam patent discloses a multiple antigen peptide that exclusively contains peptide antigen moieties. In contrast to what the Examiner states, the MAP conjugates described by the Tam '490 patent does not disclose or suggest the synthesis of conjugates containing a polysaccharide moiety. Therefore, the conjugates disclosed by the Tam '490 patent are in no way related to Applicants' carbohydrate peptide conjugates which are of an entirely different conception and structure.

According to the Examiner, the Tam '490 patent allegedly discloses a T-epitope of the VP1 protein of poliovirus type 1. Applicants wish to stress that the peptide sequences listed in

Table 1 consist both of T-cell antigens and B-cell antigens as can clearly be seen from the data of column 7, lines 54 to 56 wherein it is stated that the antigens E and F are T-cell antigens and antigen G is a B-cell antigen. There is no information in the Tam '490 patent that the poliovirus peptide antigen listed in reference K consists of a T-cell antigen. Moreover, in contrast to what the Examiner states, there is no evidence that the poliovirus antigen K of Table 1 which is also named VPg antigen is included in the VP1 protein of poliovirus. Table 1 explicitly refers to an article by Baron et al, a copy of which is submitted herewith for the Examiner's convenience, which discloses that the VPg protein is a genome linked protein able to induce a specific antibody response and the raised specific antibodies were able to react with virus specific proteins from HeLa cells infected with poliovirus. There is no information in the Baron et al reference on the T-cell antigen or the B-cell antigen nature of this peptide. The inventors contest that the disclosure of a VPg peptide in the MAP construct of Tam '490 would in any way anticipate or suggest the MAP construct of Applicants' claims.

The Examiner's statement trying to link a potential similarity between the conjugates of Tam '490 and Applicants' claimed carbohydrate peptide conjugate is totally erroneous and would be useful for supporting the alleged lack of inventive step of the claimed compounds.

With respect to the Tam '563 patent, this discloses essentially the same conjugates of the Tam '490 patent except that the Tam '563 patent describes conjugates bearing a lipophilic membrane anchoring moiety for the MAP construct to be incorporated in a liposome membrane before its administration to the body. The technical problem being solved by Tam '563 is the use of MAP conjugates in the absence of any adjuvant of immunity. This technical problem being solved by Tam '563 is effected by synthesizing MAP construct comprising a lipophilic moiety to enable the incorporation of the conjugates into liposomes for a better presentation of the different antigen determinants to the immune system.

The Tam '563 patent strengthens the demonstration of the lack of obviousness of Applicants' compounds with regard to the teachings of the Lett et al reference since the disclosure of Tam '563 further underlines that one skilled in the art at the time of Applicants' invention believe that the MAP conjugates would be effective only when used in combination with an adjuvant of immunity or under a form associated with liposomes. It can be observed from the Tam '563 patent, which is presented as a substantial improvement of the MAP construct disclosed in the Tam '490 patent, and it flows that one skilled in the art has to acknowledge that both Tam '490 and '563 patents and Lett et al would have been strongly inclined to use conjugates only under a form associated with liposomes when such conjugates are used

without any adjuvant of immunity.

The arguments of obviousness alleged by the Examiner with respect to the two Tam patents are essentially directed to the fact that the MAP constructs of Tam may contain several B- and T-epitopes on the same construct. The Examiner then concludes that the grafting of one or several B-cell epitopes on a multiple antigen peptide conjugate would be known in the prior art as applied to the construct disclosed by Lett et al. This is a completely erroneous reasoning since the technical problems to be solved by the Tam patents and in Lett et al are radically different. It was known for a long time in the prior art that polysaccharide moieties and specifically polysaccharide moieties from bacterial or yeast origin were unable by themselves to induce any specific immune response such as the production of detectable levels of specific antibodies. This is clearly stated by Lett et al in the left-hand column of page 2645 wherein it is stated "Indeed, polysaccharides are known to be poor immunogens, i.e. they induce a weak and short protection on account of their T-cell independence and consequently, their failure to induce a memory." This problem of weak immunogenicity of polysaccharide antigens is not at all related to the Tam patents which are exclusively directed to the synthesis of conjugates containing uniquely peptide antigen determinants. Therefore, this combination of prior art completely fails.


The tertiary Fung et al reference does not overcome the deficiencies of the primary and secondary references. Fung et al describes a glycoconjugate designated as S-tag consisting of multiple copies of a polysaccharide antigen (TF hapten) that has been coupled to the ALH carrier protein which has been recognized for a long time as a model for coupling a wide variety of antigens against which a specific immune response is sought.

The high molecular weight of the KLH protein as well as its large size has allowed to test for immunization of laboratory animals against hapten peptides that are not immunogenetic by themselves. Conjugates using KLH as a carrier protein induce a strong immune response against the carrier protein itself. It is not the case with construct of radical or in different conception represented by MAG constructs of Applicants' invention wherein the different antigenic moieties are grafted to a low molecular weight polylysine skeleton endowed with weak immunogenicity or even of any immunogenicity. The Examiner is in error when he combines such radially different teachings such as Fung et al and Lett et al and it must be stressed that the induction of a specific immune response is of great complexity and to date, had not been fully understood by those skilled in the art. One with the knowledge of Fung et al and Lett et al would never deduce that the grafting of the synthetic tumor associated glycoconjugate of Fung et al could have been successfully used when grafted with the conjugates disclosed by Lett et al and therefore, this combination of the

prior art fails.

In view of the amendments to the specification and claims and the above remarks, it is believed that the claims clearly point out Applicants' patentable contribution and favorable reconsideration of the application is requested.

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CAM:ds
Enclosures

Immunogenicity of Polysaccharides Conjugated to Peptides Containing T- and B-Cell Epitopes

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To develop a general model of polysaccharide-peptide vaccine, we have investigated the efficiency of linear peptides derived from protein SR, an adhesin of the I/II protein antigen family of oral streptococci, to act as carriers for two T cell-independent polysaccharides: serogroup f polysaccharide from *Streptococcus mutans* OMZ 175 (poly f) and *Saccharomyces cerevisiae* mannan. Peptide 3 (YEKEPTPTPTPDQ) and peptide 6 (TPEDPTDPTDPQDPSS), accessible on the native SR protein as demonstrated by their reactivity in enzyme-linked immunosorbent assays with rat antisera raised against protein SR, correspond to immunodominant regions of SR. Peptide 3 contains at least one B- and one T-cell epitope, as demonstrated by its ability to induce peptide- and SR-specific antibody responses without any carrier and to stimulate the proliferation of rat lymph node cells primed either with free peptide or native SR, whereas peptide 6 contains only B-cell epitope(s). Peptide 3 was then covalently coupled through reductive amination to either poly f or mannan, and peptide 6 was coupled to poly f. Subcutaneous immunizations of rats with poly f-peptide 3 or mannan-peptide 3 conjugates produced a systemic immunoglobulin M (IgM) and IgG antibody response, and the elicited antibodies reacted with free poly f or mannan, peptide 3, protein SR, and *S. mutans* or *S. cerevisiae* whole cells. Rats immunized with poly f-peptide 6 did not develop any anti-peptide or anti-SR response. Furthermore, a booster immunization of animals with poly f-peptide 3 or mannan-peptide 3 conjugates induced high titers of anti-peptide 3, anti-poly f, and anti-mannan antibodies, which occurred quickly. The response is anamnestic for the peptide and the polysaccharides and is characterized by an Ig switch from IgM to IgG. The data presented here confirm that the presence of B- and T-cell epitopes is necessary to induce an anamnestic anti-peptide response and that a peptide containing relevant B- and T-cell epitopes can act as a good carrier in improving an antipolysaccharide anamnestic immune response.

Adherence to various cells of the human body represents the first step of endogenous and exogenous infections of many pathogens (13). The specific interactions between surface-bound adhesins of microorganisms and cell surface receptors of the host are of great importance in the colonization process. Therefore, adhesins are receiving attention as vaccines which are able to induce antibodies that specifically prevent tissue colonization by pathogens. Microbial capsular and serotype polysaccharides and/or surface-bound proteins, which act as adhesins, have been tested as vaccinal antigens for numerous organisms (18). In mammals, antibody responses to polysaccharides are T cell-independent immune responses, and polysaccharides stimulate mainly immunoglobulin M (IgM) antibodies with weak memory (3). Covalent conjugation of polysaccharides to protein carriers has proved successful in overcoming this deficiency, and immunization with such conjugates often elicits a T cell-dependent antibody response to the polysaccharide (34). Proteins are generally thymus-dependent antigens which stimulate strong IgG immune responses and immunological memory. However, proteins are generally formed by multiple functional domains, only certain of which are implicated in the pathological process (i.e., adherence). The use of whole proteins as vaccine components could be hindered by the existence within the molecule of some determinants which give rise to antibodies with undesirable side

effects (i.e., autoantibodies) (9). Therefore, certain proteins might need to be detoxified, and synthetic peptides, which would eliminate many irrelevant determinants responsible for undesirable side effects, have been proposed as vaccine candidates for the production of specific antibodies against various diseases (16). It has been recognized recently that the immunogenicity of peptides is dependent on the presence of both B- and T-cell epitopes (15). In the absence of T-cell epitopes, there are two major ways of improving immunogenicity. One is to conjugate the peptide to a carrier protein (2), and the other is to conjugate the peptide to itself (32).

The choice of carrier proteins in humans has been very restricted, and in most cases polysaccharides or peptides have been coupled to tetanus toxoid or cholera toxoid because these proteins have been used in humans without untoward side effects (31). We hypothesized that an alternative to eliminating these possible undesirable side effects due to the carrier could be to use glycopeptides formed by polysaccharides covalently linked to immunogenic peptides. Because little is known about the immunogenicity of glycopeptides in inducing both anti-polysaccharide and anti-peptide antibodies, we constructed such glycopeptides. Furthermore, considering that intraspecies or interspecies cross-reactive determinants are generally important in virulence (i.e., adherence) (45), we chose to couple two widely distributed microbial polysaccharides (*Streptococcus mutans* serogroup f polysaccharide [poly f] and *Saccharomyces cerevisiae* mannan) to an immunogenic peptide derived from *S. mutans* serotype f SR protein (26), a member of the antigen I/II family of cross-reactive cell surface adhesins expressed by the alpha-hemolytic oral streptococci.

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Poly f from *S. mutans* OMZ 175 is a polymer of L-rhamnose and D-glucose formed by a polyrhamnose backbone with glucose side chains (33). We have shown previously that this serogroup polysaccharide acts as an adhesin for salivary glycoproteins and that all "mutans group" streptococci share on their cell surface determinants which cross-react with poly f. This cross-reactivity is probably due to the presence of rhamnose glucose polymers in the streptococcal cell wall (38). High levels of mannose glycans are present in the cell wall of many pathogens, including bacteria (40), mycobacteria (14), yeasts, and certain parasites (43), and in envelope glycoproteins of certain viruses (24). Proteins of the I/II family enable oral streptococci to adhere to saliva-coated cell surfaces, a property that is thought to promote streptococcal colonization of the oropharyngeal cavity (20). All these cross-reactive proteins possess a variety of common features: an amino-terminal α -helical coiled-coil structure (amino acids [aa] 50 to 600) containing a series of alanine-rich repeats, middle proline-rich tandem repeat sequences (aa 840 to 1200) with an extended structure exhibiting many β turns, and a second carboxy-terminal proline-rich wall-spanning region (20). Recently, we investigated the antigenicity and immunogenicity of six SR-derived peptides (10). Among these peptides, we chose peptide 3, a 14-residue peptide from the shared proline repeat region of SR (aa 865 to 878, 904 to 917, and 943 to 956), and peptide 6, a 16-residue peptide located in the C-terminal region (aa 1495 to 1510), for covalent coupling to polysaccharides. Our choice was influenced by the fact that both peptides are located in the salivary glycoprotein-specific adherence domain present at the C terminus of the protein SR (25), which did not correspond to the alanine-rich repeat saliva-binding domain recently defined by Crowley et al. (6) and our group (22).

In order to test the efficiency of these peptides as carriers for the induction of anti-peptide and/or anti-protein antibodies and antipolysaccharide antibodies, we first determined the presence of B- and T-cell epitope(s) in both peptide 3 and peptide 6. Next, we constructed two glycopeptides by coupling either *S. mutans* OMZ 175 poly f or *S. cerevisiae* mannan to synthetic peptides. Finally, we tested the immunogenicity of these glycopeptide conjugates to induce an anamnestic response against the peptides themselves, protein SR, and poly f and mannan.

MATERIALS AND METHODS

Animals. Male randomly outbred WISTAR rats (8 to 12 weeks old) bred in our own facilities were used for B- and T-cell epitope determination and for immunization studies with glycopeptides.

Bacterial strains and antigen preparation. *S. mutans* OMZ 175 (serogroup f) cells were grown in the D1-20 synthetic medium of Carlsson (5). Recombinant protein SR (rSR) was purified from pHbS-1-transformed *Escherichia coli* cell extract (26) by gel filtration and immunoaffinity chromatography as described by Ackermans et al. (1). Poly f antigen was prepared from lyophilized *S. mutans* cells by the method of Hamada et al. (11) and further purified according to the method of Benabdelloumène et al. (4). Mannan from *S. cerevisiae* was obtained from Sigma Chemical Co. (St. Louis, Mo.) and treated with pronase as described for poly f (4). Peptide 3 (YEKEPTPTPTPDQ) was derived from the sequence of the sr gene from *S. mutans* OMZ 175 corresponding to the center of each proline-rich repeat (aa 865 to 878, 904 to 917, and 943 to 956). Peptide 6 (TPEDPTDPTDPQDPSS) is located in the C-terminal end of the protein SR (aa 1495 to 1510) (10). Both peptides were synthesized according to the method of Merri-

field (21) by Neosystem (Strasbourg, France) and conjugated to bovine serum albumin (BSA) by using glutaraldehyde, and to ovalbumin (OVA) with bisdiazobenzidine as previously described (10).

Antiserum production. Rabbit anti-peptide 3 OVA IgG and anti-peptide 6 OVA IgG were prepared as previously described (10). Antisera against rSR, carrier-free peptide 3, carrier-free peptide 6, poly f, and mannan were raised in three rats each by four subcutaneous injections of an equal mixture of antigen (80 μ g of rSR, 80 μ g of peptide 3, 80 μ g of peptide 6, and 500 μ g of poly f or mannan in phosphate-buffered saline [PBS]) and incomplete Freund's adjuvant at 15-day intervals. Blood was collected 5 days after the last injection, and sera from control preimmune animals and from immunized animals were tested for the presence of antibodies by an indirect enzyme-linked immunosorbent assay (ELISA) using plates coated with appropriate antigens. Alkaline phosphatase (AP)-conjugated goat anti-rat Ig, anti-rat IgG, anti-rat IgA, and anti-rat IgM were purchased from Immunotech (Marseilles, France).

Determination of T-cell epitope(s). Six rats were injected at the base of the tail with 0.5 ml of incomplete Freund's adjuvant emulsion containing either 200 μ g of peptide 3, 200 μ g of peptide 6, or 100 μ g of rSR. The proliferative T-cell response was studied as described by Schneider and Vanregenmortel (37). Briefly, 8 days after immunization, the ungual lymph nodes were removed and torn in cold RPMI 1640 (GIBCO-BRL, Cergy-Pontoise, France). Fifty microliters of lymph node cells (8×10^6 cells per well) was suspended in 96-well plates (D. Dutscher, Brumath, France) and mixed with 50 μ l of RPMI 1640 supplemented with 4 mM L-glutamine, penicillin (200 IU/ml), and streptomycin (200 μ g/ml) in the presence of final concentrations of peptide 3 or peptide 6 ranging from 0.005 to 50 μ M. After 24 h at 37°C under 5% CO₂, 100 μ l of RPMI 1640 supplemented with 1% normal rat serum was added to the wells. The cultures were then incubated again under the same conditions and pulsed after 48 h with 1 μ Ci (1 mCi = 37 MBq; specific activity, 28 Ci/mmol) of [³H]thymidine (Amersham, Les Ulis, France). One-hundred-fifty-microliter samples were then harvested and spotted onto filter paper discs, dried, and counted by liquid scintillation. Titrations were performed in triplicate, and results are expressed as the stimulation index corresponding to the ratio of counts per minute of cells with peptide/counts per minute of cells without peptide.

Conjugation of polysaccharides with peptide 3 or peptide 6 and purification of glycopeptides. Poly f-peptide 3, mannan-peptide 3, and poly f-peptide 6 conjugates were prepared by the method of Sanderson and Wilson (35). Lyophilized poly f (50 mg) or mannan (50 mg) was oxidized with 10 ml of 0.1 M sodium periodate solution (pH 4.5) for 1 h at 25°C in the dark. The oxidized polysaccharides were purified by water elution on an Econo Pac 10 DG column (Bio-Rad Laboratories, Paris, France), lyophilized, and then coupled to 10 mg of peptide 3 or 10 mg of peptide 6 by reductive amination with sodium borohydride (29). Excess reagents and free peptide 3 or peptide 6 were eliminated by extensive dialysis against 0.01 M Tris HCl buffer (pH 8.0) (48 h, 4°C). The conjugates were freed from unconjugated polysaccharides by chromatography on a DEAE Trisacryl M column (1.6 by 10 cm) equilibrated with 0.01 M Tris HCl buffer (pH 8.0), and elution was performed with the same buffer containing 1 M NaCl. Conjugates were then dialyzed against PBS (24 h, 4°C) and stored at -20°C until use (27). Covalent linkages between poly f or mannan and peptide 3 and between poly f and peptide 6 were determined by a heterologous two-site sandwich ELISA pro-

cedure as described below. On the basis of the results of hexose and protein determinations, the molecular ratios of poly f to peptide 3 and mannan to peptide 3 in the glycopeptides were 1/5 and 1/50, respectively. For the poly f-peptide 6 conjugate, the ratio was 1/8.

Immunization schedule. Seven groups of six rats were immunized subcutaneously on days 0, 15, 30, and 45 with 0.5 ml of the appropriate immunogen: poly f-peptide 3 conjugate (68.5 μ g/80 μ g), mannan-peptide 3 conjugate (190 μ g/80 μ g), poly f-peptide 6 conjugate (249 μ g/80 μ g), poly f (68.5 μ g), mannan (190 μ g), free peptide 3 (80 μ g), and free peptide 6 (80 μ g). Following the first round of immunizations, rats were given booster immunizations on days 105 and 120 with the same amount of antigen. All antigens were suspended in incomplete Freund's adjuvant ensuring that equal amounts of free and conjugated polysaccharides as well as free or conjugated peptide 3 or peptide 6 were injected into the corresponding group of rats. Blood samples were harvested every 8 days from day 35 to 104 and from day 110 to 149. Each sample was tested individually for the presence of antipolysaccharide, anti-peptide 3, and anti-peptide 6 antibodies.

ELISA procedures. The presence of IgM, IgG, and IgA antibodies to poly f, mannan, peptide 3, peptide 6, protein rSR, and to whole *S. mutans* and *S. cerevisiae* cells in sera was determined by an indirect ELISA (10). Microtiter plates (Nunc; Poly Labo, Strasbourg, France) were coated with 50 μ l of either poly f (100 μ g/ml), mannan (50 μ g/ml), peptide 3 (1 μ g/ml), peptide 6 (1 μ g/ml), protein rSR (1 μ g/ml), *S. mutans* whole cells (10^9 bacteria per ml), or *S. cerevisiae* whole cells (10^9 yeast cells per ml) in 0.1 M carbonate buffer (pH 9.6). After overnight incubation at 4°C, the plates were blocked with PBS containing 0.05% Tween 20 (PBST) and 0.5% gelatin. Fifty-microliter serial dilutions of each individual serum were added to the wells (1 h, 37°C). After a washing with PBST, antibody binding was detected with 50 μ l (1/5,000) of either AP goat anti-rat IgM, AP goat anti-rat IgG, or AP goat anti-rat IgA (1 h, 37°C). After incubation (1 h, 37°C) with 50 μ l of AP substrate (*p*-nitrophenylphosphate), the A_{405} was read with an Anthos Labtec spectrophotometer. Each assay was run in triplicate and run simultaneously with preimmune sera. Antibody titers were expressed as the reciprocal (in log₂ units) of the highest serum dilution which gave an absorbance twice that of the preimmune control.

The presence of anti-peptide 3 and anti-peptide 6 antibodies in the sera of rats immunized with either rSR, free peptide 3, or free peptide 6 was assessed as described above on plates coated with peptide 3 or peptide 6 and AP goat anti-rat Ig (1/10,000).

Covalent linkage between poly f or mannan and peptide 3 or poly f and peptide 6 was checked by a heterologous two-site sandwich ELISA procedure as previously described by Wachsmann et al. (44). Briefly, 50- μ l serial dilutions of either poly f-peptide 3, mannan-peptide 3 or poly f-peptide 6 conjugate were added to wells coated with 50 μ l of rabbit anti-peptide 3 OVA IgG or rabbit anti-peptide 6 OVA IgG (50 μ g/ml). After incubation (1 h, 37°C), 50- μ l aliquots of rat anti-poly f or rat anti-mannan IgG (50 μ g/ml) were added to the wells (1 h, 37°C), which were then washed with PBST before the addition of AP-conjugated goat anti-rat IgG (1/5,000) and AP substrate.

Analytical methods. The peptide content of each conjugate was determined by the Micro BCA method (Pierce, Rockford, Ill.) with BSA as a standard. Total hexose was assayed by the resorcinol-sulfuric acid method of Monsigny et al. (23), with glucose or mannose as a standard. Molecular size determination of poly f and mannan was performed with a Waters high-performance liquid chromatography system equipped

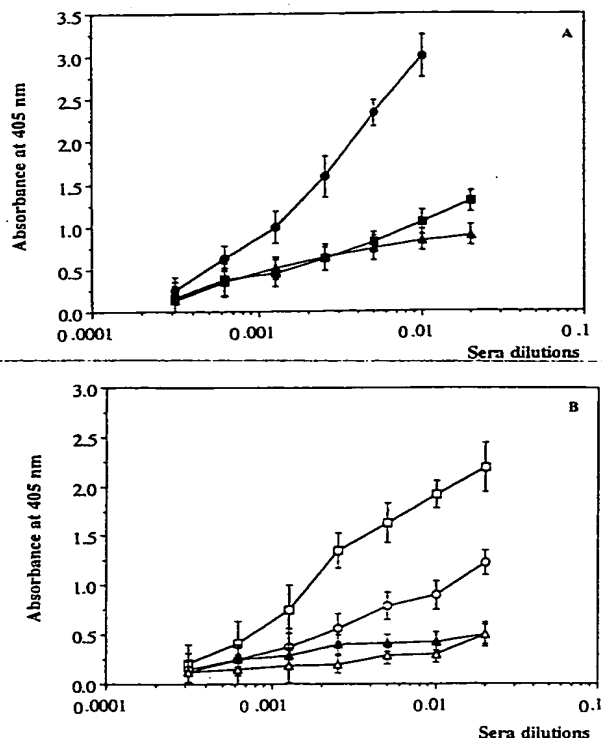


FIG. 1. (A) Reactivity of anti-rSR antibodies with rSR (●), peptide 3 (■), and peptide 6 (▲). (B) Reactivity of anti-peptide 3 antibodies with rSR (○) and peptide 3 (□), and reactivity of anti-peptide 6 antibodies with rSR (▲) and peptide 6 (△). The binding activity is expressed as the arithmetic mean of A_{405} of triplicate determinations.

with a refractive index monitor. Analysis was performed with a PAK 300 column (7.5 mm by 30 cm) with water as the eluting agent, and the column was calibrated with dextrans of different molecular sizes (Pharmacia, LKB, Uppsala, Sweden). Molecular sizes were 60,000 for poly f and 600,000 for mannan.

RESULTS

Determination of B- and T-cell epitopes on peptides. To determine the presence of B-cell epitope(s) within peptide 3 and peptide 6, we first tested the reactivity of free peptide 3 and peptide 6 against rat anti-rSR serum. As shown in Fig. 1A, peptide 3 and peptide 6 bound antibodies from rSR-immunized rats, demonstrating the presence of B-cell epitope(s) within these peptides. Secondly, we tested the abilities of peptide 3 and peptide 6 to induce a specific antibody response in rats without any carrier. Sera from all animals immunized with free peptide 3 contained antibodies which reacted in ELISA with both peptide 3 and rSR antigen (Fig. 1B), whereas animals immunized with peptide 6 failed to produce antibodies reactive with peptide 6 or rSR. In controls, rSR, peptide 3, and peptide 6 were nonreactive with preimmune rat sera and AP-conjugated anti-rat Ig. Figure 1B shows the results from one rat each immunized with peptide 3 or peptide 6 and reflects the pattern of reactivity seen for all immunized rats. Thus, both peptides contain at least one linear B-cell epitope present in the recombinant full-length protein, and peptide 3 probably contains T-cell epitope(s) that function to provide help for B cells.

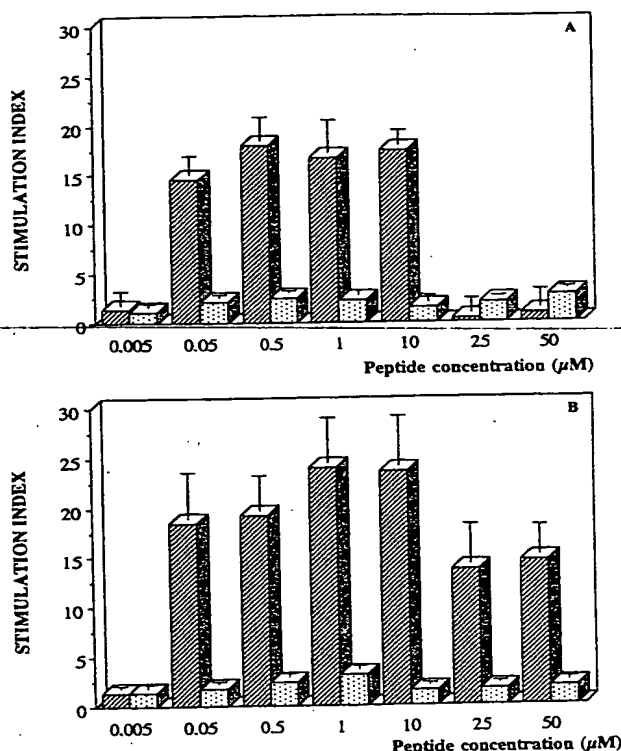


FIG. 2. (A) T-cell proliferative response to peptide 3 and peptide 6 of rats immunized and challenged with either peptide 3 (▨) or peptide 6 (□). (B) T-cell responses of rats immunized with rSR and challenged with peptide 3 (▨) or peptide 6 (□). The T-cell response to peptide 3 and peptide 6 was assessed by [3 H]TdR incorporation. Results are represented by a stimulation index and as the means of triplicate determinations for each animal. Medium background for each group of immunized animals ranged from 350 to 450 cpm.

In order to confirm the presence of T-cell epitope(s), we studied the *in vitro* proliferative response of lymph node cells from rats primed with either free peptide 3, free peptide 6, or rSR. Lymph node cells from rats primed *in vivo* with free peptide 3 and peptide 6 were challenged *in vitro* with the same peptide. Significant proliferative responses were observed with peptide 3 at doses of peptide above 0.05 μ M, peaking at 0.5 μ M (Fig. 2A). Peptide 6 failed to elicit a significant proliferative response. These results confirm the presence of T-cell epitope(s) within peptide 3 and the absence of such an epitope(s) in peptide 6. Next, we measured the proliferative response to peptide 3 or peptide 6 in animals primed with rSR (Fig. 2B). The results showed that challenge with peptide 3 induced a strong proliferative dose-dependent response similar to that observed in animals primed with free peptide 3 and that such a response was absent in animals challenged with peptide 6, confirming the presence in peptide 3 of T-cell epitope(s). Lymph node cells from rats immunized with PBS and incomplete Freund's adjuvant showed no proliferative response above background level (data not shown), indicating that peptide 3 does not activate rat T cells polyclonally. Taken together, these data suggest that peptide 3 and peptide 6 each contain at least one linear B-cell epitope which is present in the native molecule. Only peptide 3 elicited a T-cell response suggesting that peptide 3 also contains a T-cell recognition site

which is also recognized on the full-length recombinant SR molecule.

Primary antibody response to immunization with polysaccharide-peptide conjugates. The ability of peptides 3 and 6 to act as carriers for two thymo-independent antigens, poly f and mannan, was tested after covalent linkage of peptide 3 and peptide 6 to poly f and peptide 3 to mannan. The conjugation procedure, which consists of linking carbonyl groups of oxidized polysaccharides to amino groups of peptides, was chosen because of the fact that mild periodate oxidation of polysaccharides had little effect on their antigenicity (44, 45). During and after primary immunization with the appropriate antigens, sera of individual rats were screened periodically from day 35 to 104 by ELISA for the isotype distribution of antibodies reactive against (i) peptide 3, (ii) poly f or mannan, (iii) rSR, and (iv) whole *S. mutans* or whole *S. cerevisiae* cells. The kinetics of the IgM and IgG antibody response of rats immunized with poly f-peptide 3 are depicted in Fig. 3A to D. Rats immunized with poly f-peptide 3 elicited a strong IgM response and a weaker IgG response against peptide 3, similar to the response obtained with rats immunized with free peptide 3 (Fig. 3A), which declined over a 37-day period. Immunization of rats with poly f-peptide 6 did not induce any serum IgM or IgG response to peptide 6 (data not shown). Both peptide 3-poly f and peptide 6-poly f conjugates also induced an anti-poly f IgM response comparable to the response obtained after priming with free poly f (Fig. 3B); however, only poly f-peptide 3 elicited a weak anti-poly f IgG response (Fig. 3B). Furthermore, priming of rats with poly f-peptide 3 induced antibodies which reacted with rSR (Fig. 3C) and whole *S. mutans* cells (Fig. 3D) and the reactivity of the immune sera against both antigens revealed a pattern similar to those of the IgM and IgG antibody responses. No responses against rSR and *S. mutans* cells were detected in rats primed with poly f-peptide 6 (data not shown). Anti-peptide or anti-poly f IgA antibodies were never detected in immune sera (data not shown). A similar pattern of antibody distribution and reactivity was observed for rats primed with mannan-peptide 3. This conjugate induced IgM and IgG antibodies reactive with peptide 3 (Fig. 4A), mannan (Fig. 4B), rSR (Fig. 4C), and *S. cerevisiae* cells (Fig. 4D) and, as expected, the IgM titers were high compared with the IgG titers.

Secondary antibody response to polysaccharide-peptide conjugates. We then evaluated the abilities of poly f-peptide 3, poly f-peptide 6, and mannan-peptide 3 to induce a secondary antibody response. Rats previously immunized with either conjugates, free peptide 3, free peptide 6, or free polysaccharides were allowed to rest for 60 days after the last injection of the primary immunization and then were challenged twice with the same amounts of appropriate antigens on days 105 and 120. Rats given boosters of poly f-peptide 3 or mannan-peptide 3 showed high titers of anti-peptide 3 IgG antibodies and low titers of anti-peptide 3 IgM antibodies comparable to those obtained with free peptide 3 (Fig. 3A and 4A), whereas no anamnestic response was obtained from rats given boosters of poly f-peptide 6 (data not shown). On the other hand, only rats given boosters of poly f-peptide 3 or mannan-peptide 3 showed high titers of anti-poly f or anti-mannan IgG antibodies (Fig. 3B and 4B). Furthermore, anti-peptide 3 and anti-polysaccharide IgG responses occurred very quickly and peaked 5 days after the last booster, on day 125 (Fig. 3B and 4B), suggesting that the polysaccharide-peptide conjugates elicited an anti-polysaccharide response resembling a T cell-dependent response. The anti-peptide and the antipolysaccharide IgG antibodies produced after the booster reacted with protein rSR

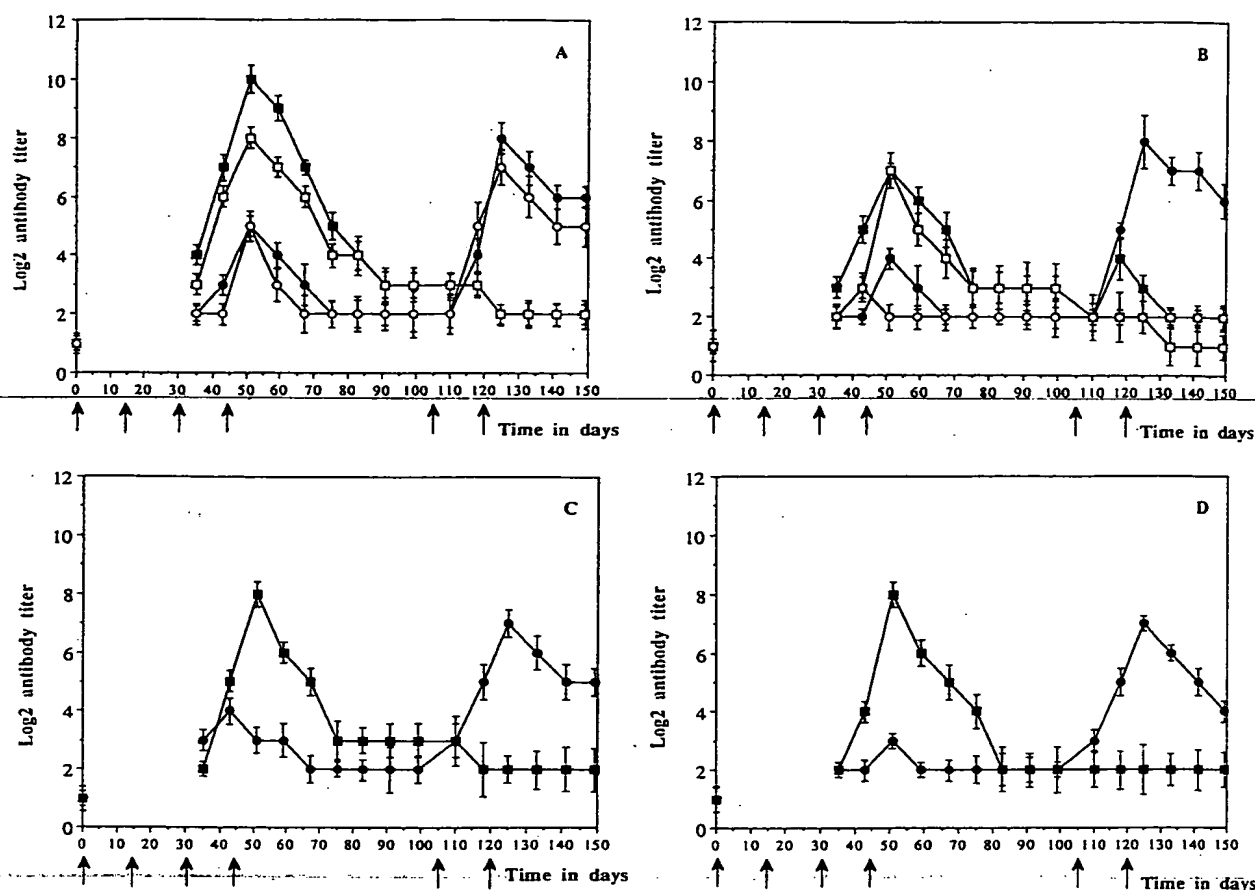


FIG. 3. IgM (■, □) and IgG (●, ○) profiles of the antibody responses against peptide 3 (A), poly f (B), rSR (C), and whole *S. mutans* cells (D) induced in rats after immunization with either poly f-peptide 3 (■, ●) (A and B) and free peptide 3 (□, ○) (A) or free poly f (□, ○) (B). Sera drawn on days 35, 43, 51, 59, 67, 75, 83, 91, and 99 for the primary response and on days 110, 118, 125, 133, 141, and 149 for the secondary response were tested individually at serial dilutions for the presence of specific IgM and IgG antibodies. Antibody titers were expressed as the reciprocal (in log₂ units) of the highest serum dilution which gave an absorbance twice that of the control. The data represent the means of triplicate determinations from the sera of six rats.

(Fig. 3C and 4C) and with whole *S. mutans* (Fig. 3D) or whole *S. cerevisiae* (Fig. 4D) cells.

DISCUSSION

In this study, we investigated the efficiency of two SR-derived peptides as carriers for a T cell-independent polysaccharide. We examined the characteristics of the humoral immune response induced by the conjugates obtained by cross-linking *S. mutans* poly f to peptides 3 and 6 via reductive amination. Similar experiments were performed with mannan from *S. cerevisiae* conjugated to peptide 3. Earlier studies showed that a good antibody response against polysaccharides could be induced only with polysaccharides coupled to protein carriers (34). The choice of the carrier, the method of coupling and the ratio of polysaccharide to carrier appear to be important parameters in the preparation of polysaccharide-protein conjugates. Therefore, in order to develop a polysaccharide-peptide vaccine we focused our interest on the carrier. We chose to use as carriers peptides derived from protein SR, an adhesin of the I/II antigen family of oral streptococci. The two previously described peptides (10) exhibit a predicted high antigenic index. Peptide 3 is located and represented more

than once in the middle proline-rich region of protein SR. Peptide 6 is located at the C terminus. After coupling to OVA, both peptides were found to be highly immunogenic in rabbits, providing a significant antibody response to the peptide and to the SR protein in its free or cell-associated form (10). Therefore, we first analyzed peptide 3 and peptide 6 for the presence of B- and T-cell epitopes by monitoring the anti-peptide humoral and cellular responses in rats. Rat anti-SR IgG was shown to bind to peptide 3 and peptide 6, suggesting that both peptides contain at least one primary sequence B-cell epitope and that this epitope is accessible on the surface of the SR molecule. However, without a carrier only peptide 3 was found to be immunogenic in rats. The anti-peptide antibodies bound to both peptide 3 and SR. The T-cell dependencies of peptide 3 and peptide 6 were further investigated. Proliferative *in vitro* assays with lymph node cells from rats immunized with peptide 3 and peptide 6 demonstrated that T cells recognized peptide 3 but not peptide 6. Furthermore, we showed that lymphocytes from rats immunized with SR recognize the epitope(s) present in peptide 3 but not that in peptide 6. Taken together, these results suggest that peptide 3, located in the adherence domain of the SR molecule (aa 800 to approximately 1200) but outside

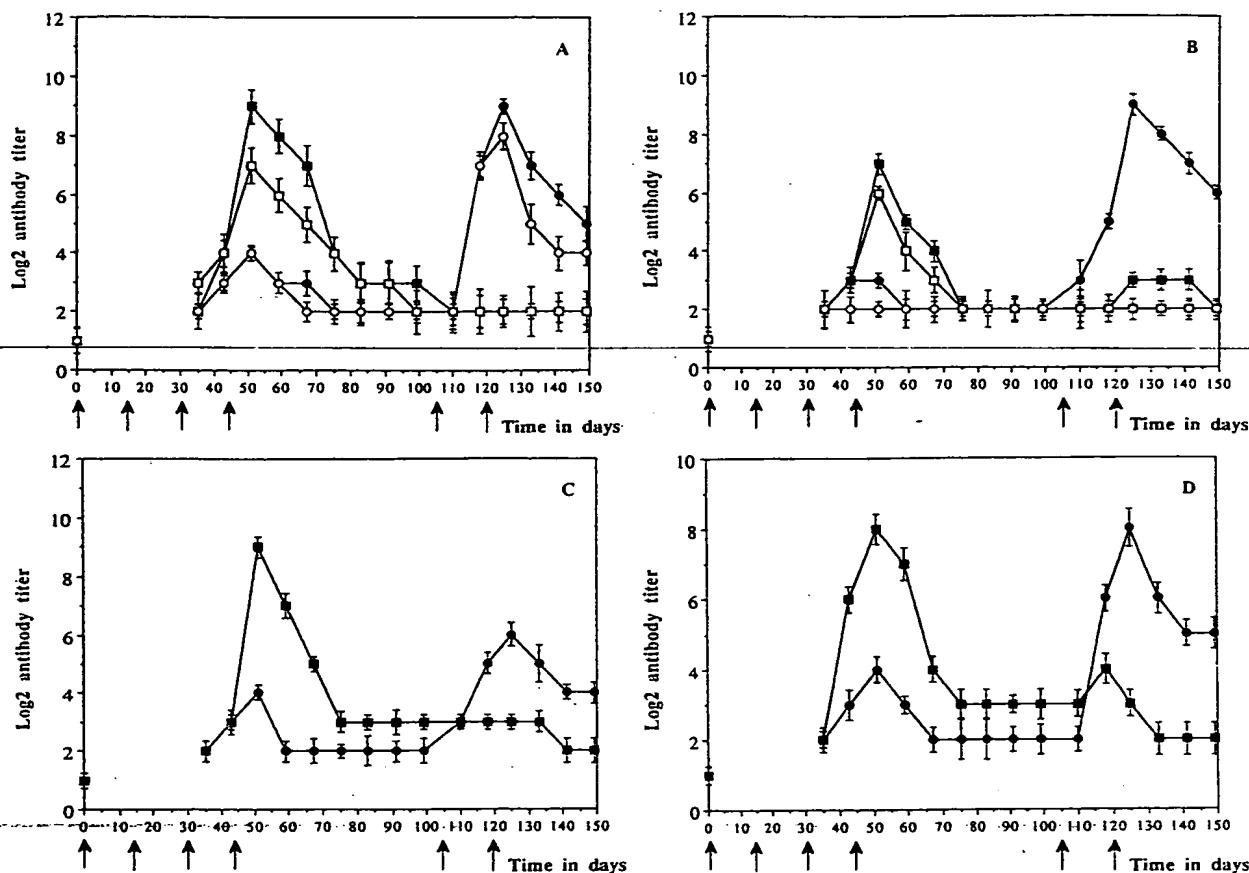


FIG. 4. IgM (■, □) and IgG (●, ○) profiles of the antibody responses against peptide 3 (A), mannan (B), rSR (C), and whole *S. cerevisiae* cells (D) induced in rats after immunization with either mannan-peptide 3 (■, ●) (A and B) and free peptide 3 (□, ○) (A) or free mannan (□, ○) (B). Sera harvested on days 35, 43, 51, 59, 67, 75, 83, 91, and 99 for the primary response and on days 110, 118, 125, 133, 141, and 149 for the secondary response were tested individually at serial dilutions for the presence of specific IgM and IgG antibodies. Antibody titers were expressed as the reciprocal (in log₂ units) of the highest serum dilution which gave an absorbance twice that of the control. The data represent the means of triplicate determinations from the sera of six rats.

of the putative human IgG cross-reactive region (aa 948 to 1028) (22), contains at least one B-cell epitope and one T-cell epitope present on the SR molecule. Peptide 6 contains only B-cell epitopes and is not immunogenic.

Poly f and mannan have been shown to be active immunomodulators for splenic lymphocytes (12, 41), demonstrating that both polysaccharides can interact with the immune system without antibody production. Despite the fact that polysaccharides are poor immunogens and that antibodies appear late in ontogeny, antibodies specific for bacterial polysaccharides are protective against various invasive bacteria (7, 17, 36), making them potential antigens against various infectious diseases. We have shown previously that the immunogenicity of poly f could be improved by chemical coupling with the *S. mutans* 74K SR protein, a maturation form of the SR molecule (44). We decided to use synthetic peptides corresponding to discrete regions of SR as carriers to improve the antigenicity of both poly f and mannan. The polysaccharides were conjugated in their high-molecular-weight form to peptide 3 and peptide 6, because polysaccharide-protein conjugates are more immunogenic than oligosaccharide-protein conjugates (30). Conformational epitope(s) is believed to be fully expressed only in high-molecular-weight forms. The choice of reductive amina-

tion as a procedure for linking polysaccharides to peptides was dictated by the fact that mild periodate oxidation of polysaccharides had little effect on their antigenic activity (44, 45). We injected rats with conjugates, keeping the dose of peptide constant, in order to determine the conjugates' ability to induce primary and secondary antibody responses to peptide and to the two polysaccharides. We showed that, as described for other peptides containing B- and T-cell epitopes, the antibody response in rats immunized with either free peptide 3, poly f-peptide 3, or mannan-peptide 3 is anamnestic and is characterized by an Ig isotype switch from IgM to IgG. Peptide 6, which contains only B-cell epitopes, did not act as a carrier to induce an immune response against the polysaccharide. We found that poly f and mannan-peptide 3 conjugates were similar in their capacities to induce anti-peptide 3 antibodies. These results are similar to those obtained by Okawa et al. (28), who showed that two peptides derived from the surface antigen of hepatitis B virus (HBsAg) conjugated to either mannan or dextran via aminocaproic acid were able to elicit an anti-peptide IgG response. Primary immunization of rats with conjugates induced a primary antipolysaccharide antibody response comparable to that obtained with free polysaccharides. The response was principally of the IgM isotype. Anti-

poly f or anti-mannan IgG antibodies were only found in sera of rats immunized with polysaccharide-peptide 3 but not polysaccharide-peptide 6 conjugates. An important observation was that rats given boosters of poly f-peptide 3 and mannan-peptide 3 conjugates exhibited a strong secondary IgG response directed against poly f and mannan. By comparing poly f-peptide 3 to mannan-peptide 3, we found that identical levels of anti-poly f or anti-mannan antibodies were induced by these conjugates. Rats given free polysaccharide boosters failed to develop any secondary antibody response to the IgG isotype. This is in contrast to the work of Okawa et al. (28), who showed that mannan and dextran-HBsAg peptide conjugates failed to elicit any antimannan or antidextran antibody response in mice. These results might be attributed to the fact that the HBsAg peptides do not contain T-cell epitopes. Therefore, it seems that the presence of both B- and T-cell epitopes is of great importance to improve the immunogenicity of polysaccharides in glycoconjugates and to improve T-helper activity. However, we cannot exclude the possibility that different presentations of the polysaccharides by using either different coupling methods or oligosaccharides could give rise to sera containing antibodies with better affinity towards polysaccharides. Furthermore, the antibody induced by poly f-peptide 3 or mannan-peptide 3 conjugates reacted with either *S. mutans* or *S. cerevisiae* whole cells, making such conjugates attractive for developing both antiprotein and antipolysaccharide antibodies.

The data presented in this paper provide evidence that a peptide containing both B- and T-cell epitopes can act as a good carrier in improving an antipolysaccharide immune response and could be used instead of whole protein. The extensive conservation of the amino acid sequence of peptide 3 among various I/II related antigens and the potential cross-reactions between mutans group polysaccharides make possible a potential vaccine against dental caries from such a conjugate. Nevertheless, recent work of Takahashi et al. (42) has demonstrated that, in mice, immune responses to a PAC-derived peptide (aa 301 to 319) are genetically restricted to major histocompatibility complex class II, suggesting that for the development of a general model of vaccine the designed peptides must also be recognized by most human leucocyte antigen genotypes. Furthermore, mannan has been implicated as a key component in bacterial or viral host interactions. The importance of high-mannose-content oligosaccharides, present on viral surface antigens such as the human immunodeficiency virus envelope gp120 (24), in virus-target interactions has been suggested by studies showing that plant lectins (19) or serum mannan binding protein (8) that recognizes certain mannan configurations reduced virus-target interactions. Therefore, antimannan antibodies could play a key role in acquired immunity against viral infections (e.g., human immunodeficiency virus) and glycopeptides obtained by conjugating oligo- or polymannosides to peptide(s) originating from the same microorganism could be an alternative to either mannoses or peptides conjugated to unrelated carrier proteins in inducing antiviral antibodies. Tetanus toxoid is generally considered an effective carrier in human vaccination, devoid of side effects. However, Schutze et al. (39) demonstrated that the immune response against synthetic peptides conjugated to tetanus toxoid can be suppressed by preexisting immunity against tetanus toxoid in a mouse model. Therefore, the use of peptides resolved (i) the choice of carriers in humans and (ii) the question of carrier-induced epitopic suppression due to the preexistence of anticarrier immunity. Further investigations are now necessary to confirm the efficiency of glycopeptides as safe synthetic vaccines against several infectious diseases.

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38-40 S: 26-22-36

Absorption 100 ml 176,00
minal) in 1% HNO₃, actual
-26-27-36/37/39

Media and Reagents
906
• 4H₂O FW 197.9
S: 26-36

100 g 102,00
500 g 269,00

100 g 73,00
500 g 273,00

0.005%
35%
b): ≤5 ppm

0.005%
01%
5%

n at 25°C: 3.5-6.0
ge 2211

RIDE SOLUTION 15 ml 169,00
laCl
7/38-40 S: 26-36-23

DE
2 FW 86.94

100 g 112,00
500 g 357,00

100 g 229,00

nic oxidations
133 (1976).

ITRATE 500 g 226,00
1 kg 383,00
An(NO₃)₂ • 4H₂O
36/37/38-40 S: 17-26-36

L-FATE
MnSO₄ • H₂O FW 169.0
5/37/38-40 S: 26-36

100 g 41,00
500 g 133,00
1 kg 226,00

e Culture Media and
1805 and
(Cont)

PRODUCT NUMBER	FF (HT)	PRODUCT NUMBER	FF (HT)
(Continuation of)			
MANGANESE SULFATE			
M 6528		Monohydrate	100 g 87,00
ACS Reagent		500 g	186,00
Assay: 98.0-101.0%		1 kg	335,00
Insoluble matter: ≤0.01%			
Loss on ignition: 10.0-12.0%			
Chloride (Cl): ≤0.005%			
Calcium (Ca): ≤0.005%			
Heavy metals (as Pb): ≤0.002%			
Iron (Fe): ≤0.002%			
Magnesium (Mg): ≤0.005%			
Nickel (Ni): ≤0.02%			
Potassium (K): ≤0.01%			
Sodium (Na): ≤0.05%			
Zinc (Zn): ≤0.005%			
Substances reducing permanganate: To pass test			
USP Grade		See: Page 2211	
MANGIFERIN			
M 3547		100 mg	191,00
(Xanthone C-glucoside)		250 mg	381,00
From Mangifera indica leaves		1 g	952,00
[4773-96-0] C₁₉H₁₈O₁₁			
FW 422.3			
R: 28 S: 45-36/37/39-22			
MANNAN			
M 7504		100 mg	110,00
From Saccharomyces		250 mg	211,00
cerevisiae		1 g	562,00
Ref.: Haworth, W.N., et al., J.		5 g	1854,00
Chem. Soc., 784 (1937).			
[9036-88-8]			
M 3640		100 mg	110,00
From Saccharomyces		250 mg	211,00
cerevisiae		1 g	562,00
Prepared by the Cetavlon method.			
Contains up to 5% ethanol.			
Ref.: Barker, S.A., et al., Chem. Ind., 330 (1957).			
[9036-88-8]			
R: 36/37/38 S: 26-36-22			
MANNAN-AGAROSE			
See under: Affinity Chromatography Media Page 1972			
MANNAN BINDING PROTEIN-AGAROSE			
See under: Affinity Chromatography Page 1996			
MANNIDE MONOOLEATE			
M 6546		5 g	94,00
Yellow to brown liquid.		25 g	297,00
A non-ionic surface active emul-		100 g	802,00
sifier.		500 g	2559,00
[9049-98-3]		2.5 kg	12093,00
MANNITE			
See: D-Mannitol Page 671			
MANNITOL SALT AGAR			
See: Microbiological Media and Components			
Page 2132			
D-MANNITOL			
(Mannite)			
[69-65-8] C₆H₁₄O₆ FW 182.2			
SigmaUltra		250 g	335,00
Residue upon ignition: <0.01%		1 kg	1117,00
Solubility (1 M in water, 20°C):			
complete, colorless			
Insoluble matter: <0.01%			
Cl: <0.05%		Mg: <0.0005%	
SO₄: <0.05%		Na: <0.005%	
AK: <0.0005%		NH₄: <0.05%	
Ca: <0.0005%		P: <0.0005%	
Cr: <0.0005%		Pb: <0.001%	
Fe: <0.0005%		Zn: <0.0005%	
K: <0.005%			
(Continued)			

PRODUCT NUMBER	FF (HT)	PRODUCT NUMBER	FF (HT)
(Continuation of)			
D-MANNITOL			
M 4125		Minimum 98%	100 g 97,00
See also: Plant Cell Culture		500 g	153,00
Reagents Page 1906		1 kg	255,00
		5 kg	1005,00
M 9647		ACS Reagent	250 g 175,00
Specific rotation [α]_D²⁵: +23.3°		500 g	297,00
to +24.3°		1 kg	591,00
Insoluble matter: ≤0.01%			
Loss on drying at 105°C: ≤0.05%			
Residue after ignition: ≤0.01%			
Titrateable acid: ≤0.0008 meq/g			
Reducing sugars: To pass test			
Heavy metals (as Pb): ≤5 ppm			
USP Grade		See: Page 2211	
D-MANNITOL-³H			
See: Radiochemicals Section Page 2184			
D-MANNITOL-1-¹⁴C			
See: Radiochemicals Section Page 2184			
MANNITOL DEHYDROGENASE			
(Mannitol: NAD oxidoreductase; EC 1.1.1.67)			
[9001-65-4]			
M 3154		From Actinobacillus sp.	10 units 516,00
Lyophilized powder		50 units	1711,00
Activity: Approx. 10 units per mg			
solid.			
Unit Definition: One unit will convert 1.0 μmole of			
D-mannitol to D-fructose per min at pH 7.6 at 37°C.			
M 9532		From Leuconostoc	5 units 516,00
mesenteroides		50 units	2852,00
Partially purified			
Lyophilized powder containing approx. 50%			
protein (Biuret); balance primarily buffer salts			
as potassium phosphate and dithiothreitol.			
Activity: 50-150 units per mg protein.			
Unit Definition: One unit will reduce 1.0 μmole of			
D-fructose per min in the presence of NADH at pH 5.3			
at 30°C.			
This preparation is useful in the determination of			
mannitol in urine. One unit of enzyme, as defined above,			
is equivalent to approximately 1.8 units of enzyme, as			
described by Lunn et al.;² one unit will oxidize			
1.0 μmole of D-mannitol per min in the presence of			
NAD at pH 8.6 at 40°C.			
Ref.: 1. Assayed according to the method of			
Yamanaka, Meth. Enzymol., 41(B), 138 (1975).			
2. Lunn, P.G., et al., Clin. Chim. Acta, 183, 163			
(1989).			
D-MANNITOL 1-PHOSPHATE			
M 4250		25 mg	290,00
Barium Salt		100 mg	790,00
White powder.		500 mg	2576,00
[104835-69-0] C₆H₁₃O₉PBa			
FW 397.5			
R: 20/22 S: 28			
Na-(1-D-MANNITYL)-L-GLUTAMINE			
See: Mannopine Page 672			
MANNOBIOSE			
See: α-D-Mannopyranosyl-D-mannopyranose			
Page 672			
D-MANNOFURANURONO-6,3-LACTONE			
See: D-Mannuronic Acid Lactone Page 675			
MANNOHEPTAOSE-DI(N-ACETYL)-			
M 6663		200 μg	1523,00
D-GLUCOSAMINE)			
(Man)₇(GlcNAc)₂; MAN-7)			
From Porcine Thyroglobulin			
Mixture of isomers			
FW 1559.4			